Original Article
The neuroprotective effects of aspirin following crush injury to rat sciatic nerve

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Abstract: Aspirin has been reported to be neuroprotective and produce some benefits for central nervous system diseases. However, the possibility of using aspirin as a neuroprotective agent for peripheral nerve injuries has rarely been reported thus far. The aim of the present study was to investigate the possibly beneficial effects of aspirin on sciatic nerve crush injury therapy in rats. Crush injury animal model was prepared with Sprague-Dawley rats. The animals were evenly divided into high-dose aspirin group, low-dose aspirin group, and vehicle group. Aspirin solution or normal saline were intraperitoneally injected once a day for 28 days after sciatic nerve crush injury. A sham-operative group was also added as normal control. The results from walking track analysis and electrophysiological assessment indicated that motor functional recovery in the aspirin groups were better than that in the vehicle group. Morphometric analysis of regenerated nerves and Fluoro-Gold retrograde tracing demonstrated that axonal regeneration in the aspirin groups was superior to that in the vehicle group. Our findings suggest that aspirin might be used as a neuroprotective agent for treating peripheral nerve injuries.

Keywords: Aspirin, neuroprotection, nerve crush injury, nerve regeneration

Introduction

During the past decades, aspirin has been reported to have various pharmacological properties and multiple sites of action [1-3]. Recently, aspirin has gained much attention because it has been shown to be neuroprotective in treating central nervous system diseases. For example, aspirin has been shown to exert protective actions against focal ischemia injury, dopamine quinone-induced neurotoxicity, and cerebral white matter lesions [4-6]. To date, neuroprotective effects of aspirin on peripheral nerve injuries have been rarely investigated.

Currently, surgical intervention, drug therapy, and physical rehabilitation are typical choices for treating peripheral nerve injuries. Among the abovementioned strategies, drug therapy is a promising approach for promoting axonal regeneration and functional recovery. Therefore, in the present study, we investigated the possibly neuroprotective effects of aspirin for promoting nerve regeneration after sciatic crush injury in rats.

Methods

Animals and surgical procedures

Male Sprague-Dawley rats (220-250 g body weight) were provided by Laboratory Animal Center of Kunming Medical University, and all animal surgeries were approved ethically by Institutional Ethical Committee of Kunming Medical University. The use of animals followed the ethical guidelines of the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

The preparation of crush injury animal model followed the protocol reported previously [7]. In brief, rats were anesthetized with an intraperitoneal injection of pentobarbital solution (1.5%, 30 mg/kg body weight). The right sciatic nerve was exposed using the gluteal muscle splitting incision under a surgical microscope. Using a pair of forceps, the exposed nerve was compressed at the proximal segment 5 mm from the bifurcation for 3 times (10 s/each time, 10 s interval). Four groups (n=9) were investigated, and aspirin solution or normal saline were intra-

peritoneally injected once a day for 28 days after surgery.

High-dose aspirin group: 10 mg aspirin (40 mg/kg body weight), given at each predefined time point. Low-dose aspirin group: 5 mg aspirin (5 mg/kg body weight), given at each predefined time point. Vehicle group: normal saline, given at each predefined time point. Sham-operative group: normal saline, the sciatic nerve was exposed without being compressed.

Subsequently, the skin was closed with 6-0 stitches and all rats were kept in animal rooms under standard housing conditions.

Behavioral analysis

Walking track analysis was performed at 7, 10, 13, 16, 19, 22, 25, and 28 days following surgery, and the sciatic functional index (SFI) was calculated according to the equation [8].

$$SFI = -38.3 \times \frac{EPL - NPL}{NPL} + 109.5 \times \frac{ETS - NTS}{NTS}$$
$$+13.3 \times \frac{EITS - NITS}{NITS} - 8.8$$

All rats were trained to walk along a standard track (100 cm long and 7 cm wide) before surgery. The hind paws were painted with red ink. Thus, changes of paw prints were recorded on white papers. The following measurements were taken from the footprints.

PL (print length): distance from the heel to the top of the third toe. ITS (intermediary toe spread): distance from the second to the fourth toe. TS (toe spread): distance between the first and the fifth toe. N (normal): for the non-operated foot. E (experimental): for the experimental foot.

The SFI value varies from -100 to 0. The value near 0 reflects normal function, while a value of approximately -100 reflects complete dysfunction.

Electrophysiological assessment

Following walking tests, animals were subjected to electrophysiological studies at 7, 13, 22 and 28 days after surgery. Under anesthesia, the right sciatic nerve was exposed as mentioned above. A bipolar stimulating electrode was applied at the proximal portion of the nerve trunk. Compound muscle action potentials (CMAPs) were recorded on the gastrocnemius belly.

Fluoro-gold retrograde tracing

Twenty-two days following surgery, retrograde labeling was performed. In brief, the right sciatic nerve was exposed and 3 µl of 4% Fluoro-gold (Biotium, Hayward, CA) solution was injected into the nerve trunk at the bifurcation by a Microliter Syringe (10 µl; Hamilton Co.). Then, the incision was sutured with 6-0 stitches and rats were return to their cages. 6 days later, the rats were intracardially perfused with paraformaldehyde (4% w/v)-phosphate buffer (0.1 M). The L4, 5, 6 segments of the lumbar spinal cord were harvested. The sample was then subjected to a standard procedure and was sectioned on a cryostat. 25 µm-thick transverse sections of the sample were then mounted on glass slides. These slides were photographed by a fluorescent microscope (BX-60; Olympus), and the number of Fluoro-gold-labeled neurons was counted by an investigator who was blinded to the experiment design.

Nerve morphometric analysis

The right sciatic nerve was removed and fixed in glutaraldehyde (3 wt%), postfixed in osmium tetroxide (1%)-sodium cacodylate buffer (0.1 M, pH=7.3). The samples were dehydrated in graded acetone, which was then replaced with acetone, and embedded in epoxy resin for sectioning. The transverse semi-thin sections (2 µm) were stained with a 1% toluidine blue/1% borax solution for light microscope. Ultrathin sections (50 nm) were stained with uranyl acetate-lead citrate for transmission electron microscope. The parameters measured included the total number of myelinated axons, the mean diameter of the nerve fibers, and the relation of axon diameter to total fiber diameter (G-ratio). All evaluations were completed by an investigator blinded to the experimental design.

Data analysis

SPSS13.0 software package (SPSS Inc., Chicago, IL) was used for statistical analysis. All data were expressed as the mean ± standard error of the mean (SEM), and were analyzed using one-way ANOVA with subsequent Bonferroni test for pairwise comparisons. $P<0.05$ were considered statistically significant.
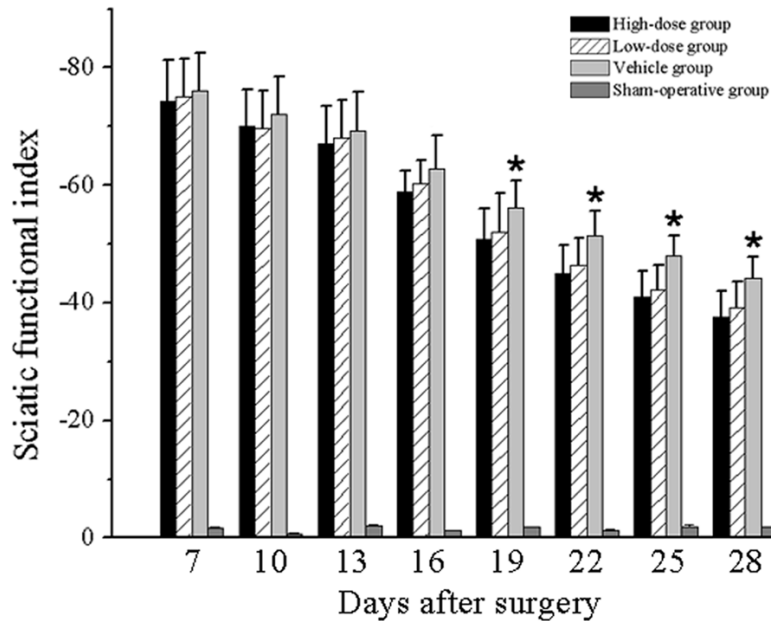
Figure 1. Sciatic functional index in all groups. *P<0.05 for comparison with the low-dose aspirin group.

Results

Effect of aspirin on motor functional recovery

SFI is commonly performed for evaluating motor functional recovery of rats with sciatic nerve injury [9, 10]. In the present study, the statistical data showed the beneficial effects of aspirin on motor functional recovery of rats after sciatic nerve crush injury. In the sham-operative group, the SFI values were near 0 at all predefined time points, reflecting normal motor function. In the aspirin groups and vehicle group, the SFI values were less than -66.98±6.48 in the early days after surgery (7, 10, 13 and 16 days), and were in similar level with no significant differences at these time points (**Figure 1**, P>0.05). Thereafter, in the vehicle group, the SFI values increased gradually, indicating a spontaneous recovery of motor function. In the later days after surgery (19, 22, 25 and 28 days), the SFI values in the low-dose aspirin group were significantly higher than that in the vehicle group (P<0.05), indicating that aspirin is beneficial for motor functional recovery. In addition, the SFI values in the high-dose aspirin group were slightly higher than those in the low-dose aspirin group in the later days after surgery without significant differences.

Electrophysiological tests were used to evaluate the extent of motor functional recovery. The data showed that, in the later days after surgery, aspirin led to a better functional recovery in rats, with higher peak amplitude of CMAP and higher nerve conduction velocity in the low-dose aspirin group than those in the vehicle group (**Figure 2**, P<0.05). In addition, the peak amplitude of CMAP and nerve conduction velocity values in the high-dose aspirin group were slightly higher than those in the low-dose aspirin group without significant differences.

Effect of aspirin on axonal regeneration

Aspirin was proved to be capable of promoting axonal regeneration in rats with sciatic nerve crush injury. 28 days after surgery, both the total number of myelinated axons and the mean diameter of the nerve fibers in the low-dose aspirin group were significantly higher than those in the vehicle group (P<0.05, **Figure 3**; **Table 1**), while these parameters in the high-dose aspirin group were slightly higher than those in the low-dose aspirin group without significant differences. Also, the G-ratio value in the low-dose aspirin group was significantly higher than that in the vehicle group, while the parameter in the high-dose aspirin group was slightly higher than those in the low-dose aspirin group without significant differences.

Fluoro-gold-labeled motoneurons appeared within the anterior horn of spinal cord of the selected segments in all groups, and were counted to identify the number of neurons that successfully outgrew into the distal branches (**Figure 4**). The percentage of Fluoro-gold-labeled neurons was significantly higher in the low-dose aspirin group than that in the vehicle group. The number of Fluoro-gold-positive neurons in the high-dose aspirin group and the low-dose aspirin group was in the similar range.
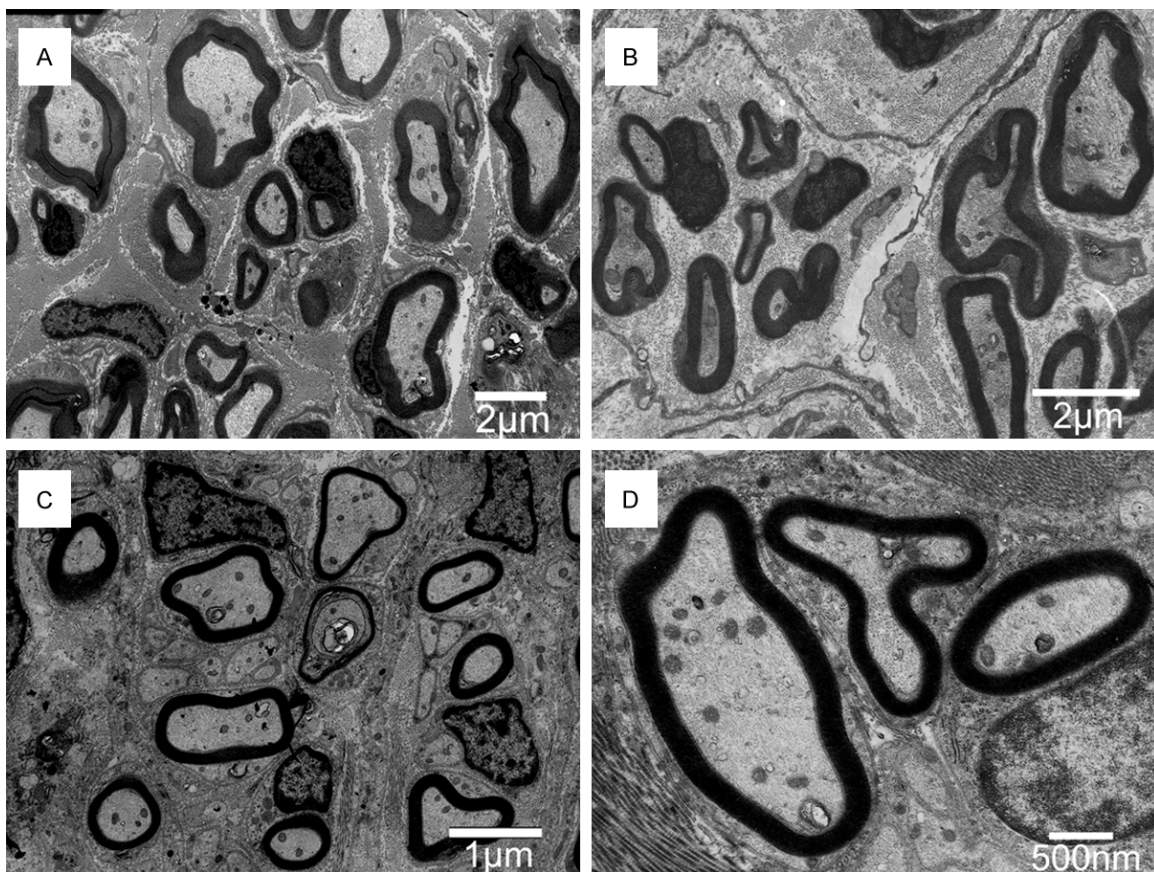
Discussion

In the present study, we analyzed the efficacy of intraperitoneally administrating aspirin on nerve regeneration in a rat model of sciatic nerve crush injury. Nerve morphometric analy-
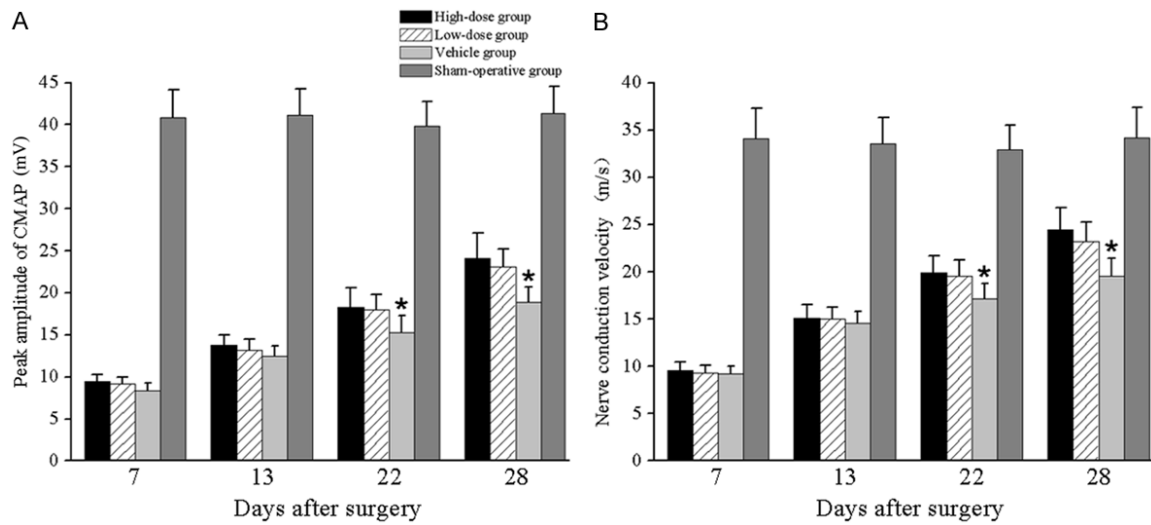
Figure 2. Peak amplitude of CMAP (A) and nerve conduction velocity (B). *P<0.05 for comparison with the low-dose aspirin group.

Figure 3. Transmission electron micrographs of regenerated nerves in the high-dose aspirin group (A), low-dose aspirin group (B), vehicle group (C), and sham-operative group (D). *P<0.05 for comparison with the low-dose aspirin group.

sis and Fluoro-gold retrograde tracing in the study showed a beneficial effect of administrating aspirin on axonal regeneration. Electrophysiological studies and walking analysis

Table 1. Morphometric values in all groups at 28 days after surgery

	High-dose aspirin group	Low-dose aspirin group	Vehicle group	Sham-operative group
Total number of myelinated axons (×10⁴)	0.345±0.021*	0.335±0.021*	0.268±0.033	0.541±0.047
Mean diameter of myelinated axons (μm)	4.020±0.383*	3.921±0.283*	3.463±0.235	4.701±0.583
Axon to fiber diameter ratio (G-ratio)	0.677±0.134*	0.682±0.104*	0.721±0.127	0.562±0.087

*$P<0.05$ for comparison with the vehicle group.
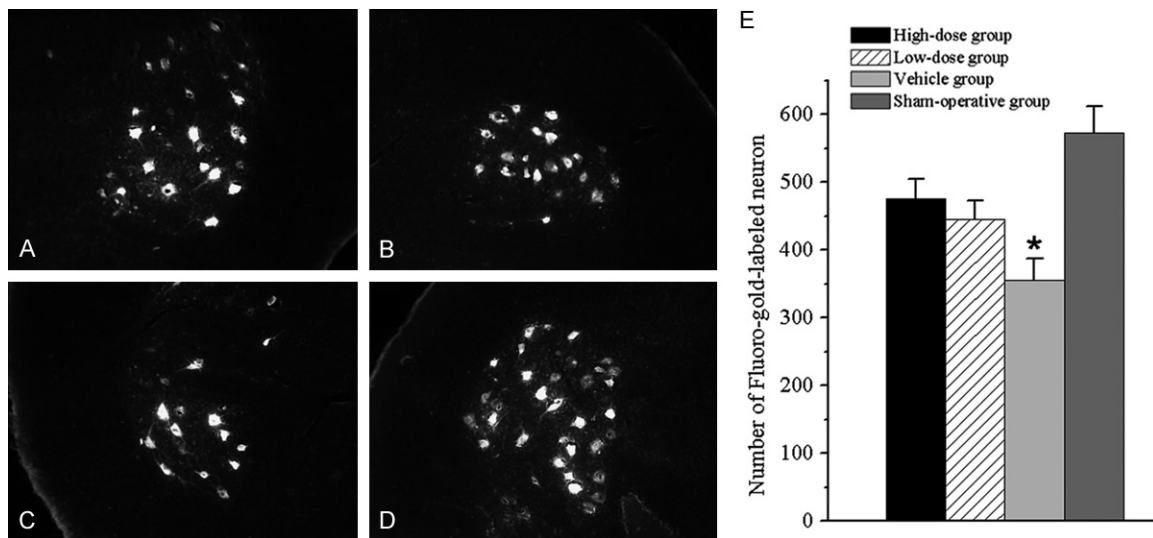
Figure 4. Fluoro-gold-labeled motoneurons in the high-dose aspirin group (A), low-dose aspirin group (B), vehicle group (C), and sham-operative group (D). The total number of Fluoro-gold-labeled motoneurons (E). *$P<0.05$ for comparison with the low-dose aspirin group. Original magnification: (A-D) ×100.

indicated indirect evidences of successful target muscles reinnervation in the aspirin-treated animals. All these results indicated that administrating aspirin led to better axonal regeneration and functional recovery of the injured sciatic nerve in rats.

During the past decade, a growing body of evidences have shown that aspirin possesses neuroprotective effects, such as protecting dopaminergic cells from dopamine quinone-induced neurotoxicity, and promoting oligodendrogenesis and oligodendrocyte myelination following cerebral white matter lesion [4, 5]. In our present study, aspirin exerted beneficial effects on promoting axonal regeneration. The number of motoneurons that survived and outgrew nerve fibers successfully achieving distal stumps was significantly higher in the aspirin treated groups than that in the vehicle group. Also, the myelin sheath and mean diameter of regenerative axons in rats with aspirin treatment were superior to those in rats without.

However, nerve fibers regenerating across the injured sites and arriving at the distal stump do not necessarily reflect functional recovery [11]. Therefore, further analyses were performed to evaluate the recovery of motor function. In the rat model of sciatic nerve injury, walking track analysis is a comprehensive and reliable index for evaluating the recovery of motor function [12]. Our results showed that the SFI values in the aspirin groups were significantly higher than those in the vehicle group. These results were concomitant with the histomorphometric improvement of regenerated nerves.

Crush injury is one of the most commonly encountered types of peripheral nerve injuries in clinical settings. Nerve crush injury is characterized by the disruption of myelin sheaths and axons following injury. Schwann cells are the primary glial cells presented in peripheral nerve system and play essential roles in axonal regeneration [13, 14]. After nerve injuries, Schwann cells start to proliferate and form Bands of

Büngner, hence providing an appropriate environment for axonal regrowth. In the later stage of regeneration, Schwann cells contribute to the remyelination of axons, which is vitally important for successful nerve regeneration. Aspirin has been reported to be beneficial for attenuating demyelination and increasing myelin thickness [4]. In addition, aspirin holds the potential to promote proliferation and differentiation of glial cells. Therefore, it is reasonable to imply that axonal regeneration and functional recovery in the present study might be attributed to the possibly beneficial effects of aspirin on Schwann cells. However, these need to be investigated by further studies.

In conclusion, the present study demonstrated that aspirin hold the potential to improve axonal regeneration and functional recovery. Thus, aspirin could be used as a potential neuroprotective agent for peripheral nerve injury therapy. Nevertheless, the mechanism underlying the neuroprotective effects of aspirin still need to be investigated by further work. In addition, the optimal dose of aspirin and the duration of use for improving functional nerve regeneration remain to be identified yet. All these issues would be addressed by future studies.

Disclosure of conflict of interest

None.

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